Recombinant acid ceramidase reduces inflammation and infection in cystic fibrosis

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Impact of this research

We demonstrate that the sphingolipid profile in cystic fibrosis (CF) airway epithelial cells is abnormal due to altered function of metabolizing enzymes leading to ceramide accumulation and a lack of upregulation of sphingosine, which has antimicrobial properties, in response to *Pseudomonas aeruginosa*. It is possible to modulate ceramide levels via treatment with recombinant acid ceramidase and demonstrate reduced susceptibility to infection and inflammation. Acid ceramidase is already being used therapeutically in people with Farber Disease and therefore has translational potential to be repurposed to ameliorate the two pivotal features of cystic fibrosis lung disease, inflammation and infection.

Abstract

Rationale: In cystic fibrosis the major cause of morbidity and mortality is lung disease characterized by inflammation and infection. The influence of sphingolipid metabolism is poorly understood with a lack of studies using human airway model systems.

Objectives: To investigate sphingolipid metabolism in cystic fibrosis and the effects of treatment with recombinant human acid ceramidase on inflammation and infection.

Methods: Sphingolipids were measured using mass spectrometry in fully-differentiated cultures of primary human airway epithelial cells and co-cultures with *Pseudomonas aeruginosa*. *In situ* activity assays, Western blotting and quantitative polymerase chain reaction were used to investigate function and expression of ceramidase and sphingomyelinase. Effects of treatment with recombinant human acid ceramidase on sphingolipid profile and inflammatory mediator production were assessed in cell cultures and murine models.

Measurements and Main Results: Ceramide is increased in cystic fibrosis airway epithelium due to differential function of enzymes regulating sphingolipid metabolism. Sphingosine, a metabolite of ceramide with antimicrobial properties, is not upregulated in response to *Pseudomonas aeruginosa* by cystic fibrosis airway epithelia. Tumor necrosis factor receptor 1 is increased in cystic fibrosis epithelia and activates NF-κB signaling, generating inflammation. Treatment with recombinant human acid ceramidase, to decrease ceramide, reduced both inflammatory mediator production and susceptibility to infection.

Conclusions: Sphingolipid metabolism is altered in airway epithelial cells cultured from people with cystic fibrosis. Treatment with recombinant acid ceramidase

ameliorates the two pivotal features of cystic fibrosis lung disease, inflammation and infection, and thus represents a therapeutic approach worthy of further exploration.

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Key words

sphingolipid; ceramide; sphingosine; lung

Introduction

Cystic fibrosis (CF) is among the most common life-limiting genetic disorders worldwide(1, 2). The major cause of morbidity and mortality in CF is lung disease characterized by neutrophilic inflammation, mucus retention and susceptibility to endobronchial infection with, in particular, *Staphylococcus aureus* and *Pseudomonas aeruginosa*(1). A cycle of inflammation and infection ensues, resulting in progressive bronchiectasis. Several hypotheses have been proposed to explain the pathophysiology seen in CF lung disease, including abnormal volume, pH and electrolyte content of the airway surface liquid(3-8). However, the precise mechanisms are not fully elucidated and multiple processes that impact on inflammation and defense against infection are likely to be involved.

Sphingolipids form membrane domains that can interact to alter the function of membrane components and modulate a diverse range of biologically important processes(9). Sphingolipids have previously been linked to CF pathophysiology. Increased levels of ceramide have been identified in the airway epithelium of CF murine models that, when normalized, reduced inflammation and susceptibility to *P. aeruginosa* infection(10, 11). Raised ceramide has also been found in the epithelium of lungs removed at the time of transplantation from people with CF(12). Reports have suggested that relative concentrations of ceramides may be important, with different chain lengths over- and under-represented in the blood of people with CF and murine models(13-15). Lipidomic analysis of bronchoalveolar lavage (BAL) fluid has recently demonstrated an altered profile in CF(16, 17).

Studies using human airway model systems are lacking, representing an important gap. In part, this is due to the fact that fully differentiated cultures of primary human

bronchial airway epithelial cells from people with CF present technical challenges (18). In the context of sphingolipid metabolism, this human model allows investigation of underlying mechanisms and refinement of targets for intervention.

Here we investigate the metabolism of ceramides using primary human airway epithelial cell cultures, BAL fluid and murine models. We describe how the function of key enzymes involved in ceramide metabolism - acid sphingomyelinase (which converts sphingomyelin to ceramide) and acid ceramidase (which converts ceramide to sphingosine) - is altered in CF. This promotes accumulation of ceramides that in turn leads to inflammation (generated via tumor necrosis factor receptor 1 (TNFR1) activation and NF-κB signaling), and susceptibility to infection (due to lack of upregulation of sphingosine). Treatment of airway epithelial cell cultures with recombinant human acid ceramidase (rhAC) decreased inflammation and infection. Furthermore, in murine models, nebulization of rhAC reduced airway inflammation, suggesting a therapeutic approach worthy of further investigation.

Methods

Primary airway epithelial cell culture and culture treatment

Primary bronchial epithelial cells were cultured at an air liquid interface (ALI) as previously described(18). Clinical characteristics of the patients studied are in Table E1 in the online data supplement. All cultures generated cilia, produced mucus and had a trans-epithelial resistance >250 $\Omega*cm^2$.

For co-culture experiments ALI cultures were transitioned to antibiotic-free medium, and $1x10^5$ colony forming units of *P. aeruginosa* (PA01) in 100 µL phosphate-buffered saline were added to the apical surface, and incubated for 24 hours. In specific experiments, cultures were treated apically for 1 hour with: 100 µL of rhAC (20 µg/mL), generated as described previously(19); the highly selective and potent cRel inhibitor IT 901 (2 µM) for 24 hours; or the CF transmembrance conductance regulator (CFTR) modulators ivacaftor or tezacaftor-ivacaftor in combination (each 5 µM) for 48 hours with dose refreshed after 24 hours.

Detailed methods, including sample preparation for analysis, and standard methodologies (Western blotting, real-time qPCR and ELISA) are provided in the online data supplement.

Analysis of sphingolipid profile of cell cultures by mass spectrometry

Calibration curves for all assayed ceramide and sphingosine species were constructed using appropriate standards. All standards and samples were analyzed in triplicate with the ABSciex QTrap 4000 system, using a 3-scan event methodology to reduce matrix noise. For selectivity the mass tolerance for each ion was set to within 0.01 m/z, which allowed for accurate quantification.

BAL ceramide measurement

For ceramide determination, a ceramide hydrolysis buffer (0.2 M citric/phosphate buffer, 0.3 M NaCl and 0.2 mg/ml of recombinant acid ceramidase) was mixed with the total lipid extract solution (1:1,v/v) and incubated at 37 °C for 1 hour. Cell-free supernatant samples were analyzed using an Acquity H-Class UPLC system equipped with a Waters Acquity UPLC BEH RP18 column.

Ceramidase and sphingomyelinase functional *in situ* assays

100 µL of buffered solution containing either BODIPY® TR Ceramide or BODIPY® FL C12-Sphingomyelin at a 1:2000 dilution was applied to the apical surface of ALI cultures. The lipid fraction was isolated as described in the online data supplement and samples were separated by thin layer chromatography with chloroform:methanol (5:1 v/v).

Mice studies

Two different *Cftr* mutant mouse strains and their respective syngeneic littermates were used. *Cftr*^{tm1Unc}-*Tg*$^{(FABPCFTR)}$ (abbreviated *Cftr*^{KO}) Jaw mice are genetically deficient for the murine equivalent to human CFTR (*Cftr*), but express human CFTR in the gut under control of a fatty acid binding protein promoter to prevent acute intestinal obstruction. B6.129P2(CF/3)-*Cftr*$^{TgH(neoim)Hgu}$ (abbreviated *Cftr*^{MHH}) congenic mice were also used that have a low residual activity of Cftr allowing normal development and feeding.

Nebulization of recombinant human acid ceramidase

Cftr^{KO} and *Cftr*^{MHH} mice were nebulized with rhAC 200 µg diluted in 800 µL 0.9% NaCl solution using Pari boy nebulizer apparatus over 10 minutes. rhAC was nebulized on 3 consecutive days when mice were 24 weeks old. The trachea was removed 6 hours after the last inhalation for further analysis as described in the online data supplement.

Results

Ceramide metabolism is dysregulated in cystic fibrosis airway epithelial cells

Mass spectrometry was used to investigate the ceramide profile in primary bronchial epithelial cells isolated from people with CF or controls, and fully differentiated at an ALI. Clinical characteristics of the patients studied are in Table E1 in the online data supplement. Under basal conditions, total levels of ceramide were increased in CF cultures (Fig 1a) with similar levels of sphingosine, a key metabolite of ceramide, in CF and non-CF cultures (Fig 1b). There was increased C16 and C22 ceramide in CF cultures (Fig 1c). In murine models, normalization of sphingolipids has been associated with reduction in susceptibility to *P. aeruginosa,* the most significant respiratory pathogen in CF(10). We therefore investigated the ceramide profile of cultures following co-culture with live *P. aeruginosa.* In both CF and non-CF cultures total ceramide was increased following exposure to *P. aeruginosa* (Fig 1a), with statistically significant increases in C16, C20 and C22 observed in CF cultures only (Fig 1c). An increase in sphingosine occurred in non-CF cultures exposed to *P. aeruginosa* (Fig 1b). However, in CF cultures *P. aeruginosa* did not induce changes. Sphingolipids are pivotal constituents of plasma membranes with enriched domains crucial for modulating cellular functions. We therefore also measured sphingolipid concentrations in plasma membrane fractions and found a similar pattern to that in whole cell lysates for ceramide and sphingosine (Figs 1d-f). The proportion of ceramide in membrane fractions was comparable across all experiments (Fig 1g). An increase in sphingosine occurred in non-CF cultures exposed to *P. aeruginosa* in plasma membrane fractions (Fig 1e) with an increase in percentage present in the plasma membrane (Fig 1h).

Similar to results in whole cell lysates, *P. aeruginosa* did not induce changes in plasma membrane sphingosine in CF cultures (Figs 1e and 1h).

Collectively, these data suggest that ceramide accumulates in human CF airway epithelial cells with a distinct profile of individual species present, including both long chain and very long chain ceramides. In response to *P. aeruginosa* non-CF cultures upregulate sphingosine, an effect not seen in CF cultures.

Ceramide is increased in BAL fluid from children and young people with cystic fibrosis

To test whether the situation *in vitro* was mirrored *in situ*, we measured total ceramide in BAL fluid collected during clinically-indicated bronchoscopies from children and young people with CF and an age-matched comparator group who do not have CF but underwent investigation for respiratory problems (clinical details are in Table E1 in the online data supplement). Levels of ceramide were increased in the CF group (Fig 2a).

In cystic fibrosis epithelia there is decreased function of ceramidase and increased function of sphingomyelinase

To investigate potential mechanisms responsible for ceramide accumulation we measured functional enzyme activity at the apical surface of cell cultures. Total ceramidase activity was reduced in CF cultures (Fig 3a). Acid ceramidase protein and *ASAH1* (coding for acid ceramidase) gene expression were decreased in CF cells compared to non-CF (Figs 3b and c). Following co-culture with *P. aeruginosa* there

was an increase in acid ceramidase protein and *ASAH1* gene expression in non-CF cultures with a smaller increase observed in CF cultures (Figs 3b and c).

Conversely, sphingomyelinase activity was increased (Fig 3d) in CF cultures. However, there was no difference in acid sphingomyelinase protein or the expression of the *SMPD1* (coding for acid sphingomyelinase) gene between any cell type or treatment (Figs 3e and f).

Together, these data suggest that a combination of reductions in expression and function of acid ceramidase (which converts ceramide to sphingosine) and an increase in sphingomyelinase (which converts sphingomyelin to ceramide) function serve to promote the accumulation of ceramide observed in human CF airway epithelium.

Treatment with recombinant human acid ceramidase reduces levels of ceramide in cystic fibrosis airway epithelia

Based on Figure 3, we assessed the capacity for rhAC to modulate ceramide levels in human airway epithelial cells. rhAC has recently been developed as an enzyme replacement therapy for Farber disease(20-22). Initial experiments established no cytotoxic effect of rhAC on airway epithelial cell cultures (Figs E2a and b in the online data supplement). A single treatment of CF cultures with rhAC reduced ceramide and restored levels close to those seen in non-CF cultures (Fig 4a). For sphingosine, no statistically significant differences were detected at the whole cell level (Fig 4b). Treatment with rhAC reduced C16, C22 and C24 ceramide (Fig 4c). In plasma membrane fractions, a similar reduction in total ceramide was observed (Fig 4d) along with an increase in sphingosine levels (Fig 4e) that was statistically significant in non-

CF cultures. At the individual species level, only C24 ceramide was statistically significantly reduced (Fig 4f). Following rhAC treatment the proportion of ceramide in the plasma membrane was unchanged (Fig 4g) and the proportion of sphingosine present in the plasma membrane in both CF and non-CF cultures was increased (Fig 4h).

Inflammatory responses are reduced following treatment with recombinant human acid ceramidase

Neutrophilic airway inflammation is a pivotal part of CF lung disease pathophysiology(1). In view of the role of sphingolipids in modulating inflammatory responses we investigated the effect of rhAC treatment on inflammatory mediator production at the apical surface of airway epithelial cell cultures(9). At baseline, increased secretion of IL-8, IL-1β and TNFα (Figs 5a-c), was observed in CF cultures, with no statistically significant differences seen in IL-4 or IL-6, (Figs E3a-b in the online data supplement). Application of rhAC resulted in significantly reduced secretion of IL-8 in CF cultures, to levels comparable with control non-CF cultures, and this effect was maintained for 5 days following a single treatment (Fig 5a). A similar, though less marked, effect of rhAC was observed for IL-1β, TNFα (Figs 5b and c) and IL-6 (Fig E3a in the online data supplement). Comparison was made with the effect of modulating CFTR function in CF cultures homozygous for F508del with ivacaftor or tezacaftor-ivacaftor. Again, treatment with rhAC reduced IL-8 production with a small, but not statistically significant, reduction seen with tezacaftor-ivacaftor and no synergistic effect with rhAC and tezacaftor-ivacftor (Fig 5d).

To further investigate the potential for rhAC as an anti-inflammatory therapy in CF, we examined the effect of nebulized rhAC on airway inflammation in two different CF murine models. *Cftr*^{tm1Unc}-*Tg*$^{(FABPCFTR)}$ (*Cftr*^{KO}) mice are genetically deficient for the murine equivalent to human CFTR (Cftr), but express human CFTR in the gut under control of a fatty acid binding protein promoter to prevent acute intestinal obstruction. In contrast, B6.129P2(CF/3)-*Cftr*$^{TgH(neoim)Hgu}$ (*Cftr*^{MHH}) congenic mice have a low residual activity of Cftr allowing normal development and feeding. Increased numbers of neutrophils and macrophages were observed in the lungs of CF mice (Figs 6a and b). This was associated with increased ceramide levels (Fig E4a in the online data supplement). Nebulization of rhAC to mice daily for 3 days reduced neutrophil and macrophage numbers towards wild type levels (Figs 6a and b).

These data suggest that treatment with rhAC reduces the production of several key pro-inflammatory cytokines and chemokines by CF airway epithelial cell cultures. Furthermore, nebulization of rhAC in two different murine models was associated with a reduction in cellular markers of lung inflammation.

Inflammatory responses are driven by altered tumor necrosis factor receptor 1 expression and NF-κB activation

Changes in the lipid composition of plasma membranes can significantly alter receptor expression and downstream signaling events(11, 23). Sphingolipid-enriched membrane domains have been shown to be essential for TNFR1 activation and subsequent NF-κB signaling(24). We investigated TNFR1 expression in CF cultures and found it to be increased compared to non-CF cultures (Figs 7a and b). This reduced following treatment with rhAC (Figs 7a and b). These findings were confirmed

by Western blotting for TNFR1 in plasma membrane fractions (Fig E5 in the online data supplement). To investigate downstream signaling from TNFR1 we investigated expression and nuclear localization of NF-κB subunit cRel (Figs 7c and d). Co-culture with *P. aeruginosa* resulted in an increase in the nuclear localization of cRel in CF cultures. Treatment with rhAC increased cytoplasmic cRel, with a corresponding reduction in nuclear localization of cRel (Figs 7c and d). Use of a specific cRel inhibitor reduced IL-8 production (Fig 7e). In human airway tissue sections (clinical details are shown in Table E1 in the online data supplement), significantly more cRel was localized in the nucleus of epithelial cells from people with advanced CF lung disease compared to unused donor lungs (Figs 7f-h). Due to TNFR1 also being expressed at the basolateral membrane of airway epithelial cells we measured TNFα in the basolateral medium in the experiments shown in Fig 5c. Levels of TNFα were higher in the basolateral medium than in the apical washes (Fig E3c).

These data suggest that CF epithelia have increased TNFR1 expression that reduces following treatment with rhAC and the decrease in ceramide levels. Consequently, enhanced NF-κB activation is observed in CF epithelia with increased nuclear localization of cRel, which also reduces with rhAC treatment.

Treatment with recombinant human acid ceramidase reduces susceptibility to infection

Previous work has shown that reduction of ceramide in the airways of CF mice is associated with a reduction in susceptibility to infection(10, 11). We therefore examined effects of rhAC treatment on infection in primary human airway epithelial cells using two different methods.

Firstly, heat-killed, fluorescently labeled *S. aureus* were added to the apical surface of differentiated cultures. Increased numbers of *S. aureus* remained adherent to the surface of CF cultures, suggestive of an increased susceptibility to colonization (Figs 8a and b). Treatment with rhAC reduced the adherence of *S. aureus* to CF cultures (Figs 8a and b).

Secondly, we investigated defense against live *P. aeruginosa.* Increased viable bacteria were isolated from the surface and lysates of CF cultures (Figs 8c and d). Treatment with rhAC reduced the number of viable *P. aeruginosa* recovered from the apical surface of cultures (Fig 8c), while no significant reduction in bacteria internalized by CF cultures was observed (Fig 8d).

Discussion

Through mass spectrometry analysis of differentiated primary airway epithelial cell cultures we have shown, and quantified, an altered ceramide profile in human CF epithelia. Increased ceramide was also detected in BAL fluid from children and young people with CF. This supports previous observations made in some murine models and agrees with immunohistochemistry performed on the bronchial epithelium of explanted advanced CF lung disease tissue (10, 12, 13).

Previous studies of sphingolipids in CF, using different models and techniques, have found varying results(15). In homogenized explanted lung tissue (containing multiple cell types and representing end-stage disease) increased C16, C18 and C20, but not

C22, ceramide species were observed(12). In contrast, a cell line model transfected with an antisense *CFTR* construct demonstrated reduced levels of C18, but increased levels of C22, C24 and C26(25). Findings in another CF murine model and the peripheral blood of people with CF have suggested a reduction in C24 ceramide and increase in C16(13, 15). Here, using a fully differentiated model in primary human epithelial cells we found increased C16 and C22 in whole cell lysates and increased C24 in the plasma membrane. These ceramide chain lengths and their metabolites are known to be involved in inflammation and apoptosis(9, 12, 15, 26-31).

Data presented here suggest that ceramide accumulates in CF airway epithelia due to differential function of enzymes. Decreased ceramidase function combined with increased sphingomyelinase activity favor increased ceramide accumulation. Western blot and gene expression analysis further showed that the decreased ceramidase activity was due to reduced acid ceramidase expression. This agrees with work in CF murine models showing that β1-integrin is trapped in the apical membrane of airway epithelial cells, downregulating acid ceramidase expression(11).

In the case of acid sphingomyelinase we found expression to be unchanged in CF epithelia. The reason for the altered activity of acid sphingomyelinase remains uncertain. Under *in vitro* conditions acid sphingomyelinase is a much more active enzyme than acid ceramidase (i.e., has a 10-fold or greater capacity to hydrolyze sphingomyelin compared with the ability of acid ceramidase to hydrolyze ceramide)(32). One possible explanation for reduced activity is that acid sphingomyelinase function is known to be pH dependent (activity increases as pH lowers to an optimum of around 5)(33, 34). The pH of the airway surface liquid in CF remains keenly debated, however, there is some evidence to suggest that homeostasis

is disordered and that the pH is lowered(3, 4, 35-38). Another potential explanation, is that acid ceramidase and acid sphingomyelinase are known to exist in a complex with inter-connected functions(19). It is therefore possible that reduced acid ceramidase expression may lead to a conformational change in acid sphingomyelinase and further enhanced enzyme activity.

Treatment of CF cultures with rhAC decreased ceramide close to levels present in non-CF cultures and reduced secretion of inflammatory mediators. Nebulization of rhAC in CF mouse models also reduced lung inflammation. We found evidence of neutrophilic inflammation in the lungs of *Cftr*^{KO} and *Cftr*^{MHH} mice. A constitutive increase in expression of IL-1 and the mouse homolog of IL-8, keratinocyte-derived chemokine, has been shown in lung homogenates from these mice (10). Reduction of ceramide levels (genetically by crossing with acid sphingomyelinase knock out animals or pharmacologically via amitriptyline treatment) has also been shown to lead to a reduction in neutrophilic inflammation(10). Our work would have been strengthened by measurement of key cytokines and chemokines in the murine lung, histological assessment of neutrophil and macrophage distribution, and by studying *in vivo* responses to airway infection.

There are divergent findings in the literature around the existence of a pro-inflammatory state in the CF airway in the absence of identifiable infection, with some evidence to support this concept from BAL studies in young children but varied findings in other studies and animal models(1, 39-44). In our primary human airway epithelial model we found increased production of several pro-inflammatory mediators in cultures derived from adults with advanced CF lung disease. The effects of altered membrane microdomain abundance on relative fluidity and stability of transmembrane

receptors involved in inflammatory responses and activity of ion channels remains poorly understood(15, 45, 46). Notably, Abu-Arish *et al.* recently demonstrated that epithelial cells respond to secretagogues by forming clusters of CFTR in ceramide-rich membrane microdomains via an acid sphingomyelinase-dependent mechanism to increase transepithelial secretion(47). We found that reducing ceramide in CF epithelial cells, via rhAC treatment, was associated with reduced TNFR1 expression, decreased cRel nuclear localization and less IL-8 production. It is recognized that activation of NF-κB results in IL-8 production, however, epigenetic factors and mRNA stability also influence this process and how a change in sphingolipid profile impacts on these is yet to be elucidated (48-50).

Relatively low levels of TNFα, in the range of 1-2 pg/mL in apical washings and 5-9 pg/mL in basolateral medium, were measured in unstimulated cultures, in keeping with those reported in the literature (51). However, epithelial cells are not the only source of TNFα in the CF lung, with reports of production by macrophages and neutrophils (52). Several studies have measured levels of TNFα in airway samples from people with CF. For example, mean levels of 130 and 400 pg/mL have been measured in sputum and BAL fluid respectively, with higher concentrations during pulmonary exacerbations(52-54).

We also found increased susceptibility to infection in CF cultures that was reduced by rhAC treatment. Sphingosine, which is generated from ceramide by acid ceramidase, is known to have antimicrobial properties and has been found to be deficient in the airway of CF murine models(11). We did not detect differences in sphingosine between CF and non-CF human cultures at baseline. However, unlike non-CF cells, on exposure to *P. aeruginosa* CF epithelial cells did not upregulate levels of sphingosine

in the plasma membrane. We propose that this represents a potentially important host-defense mechanism, dysfunction of which may contribute to the susceptibility to respiratory infection seen clinically in people with CF. Treatment with rhAC did increase plasma membrane sphingosine in cell cultures but this effect was only statistically significant in non-CF cultures. A potential explanation for this is that sphingosine produced following rhAC treatment may be rapidly metabolized. In a mouse model of Farber disease intraperitoneal rhAC treatment markedly reduced ceramide levels but sphingosine increased to a lesser extent, especially so in the lung compartment(22). When considering the proportion of sphingosine in the plasma membrane there was a significant increase in both CF and non-CF cultures with rhAC treatment. This localized increase in sphingosine may account for the effect of rhAC treatment on reducing bacterial adherence to the apical plasma membrane, but not internalization.

Limitations of our infection work are that we used a laboratory strain of *P. aeruginosa* rather than a clinical isolate and did not work with live *S. aureus.* Proof of principle data were generated to investigate the effects of acute rhAC treatment on inflammation and infection. To advance along a translational path further evidence will likely need to be generated for longer-term treatment and efficacy in larger animal models prior to experimental medicine studies in humans.

Collectively, our work supports the concept that disordered sphingolipid metabolism is involved in CF lung disease pathogenesis - linking to both inflammation and infection. Restoring acid ceramidase activity with rhAC treatment therefore represents an intriguing novel potential approach to target these two key pathological processes in the airways of people with CF. Despite exciting developments in the field of CFTR modulators there remains an unmet need to develop therapies that ameliorate ongoing

problems with inflammation and infection(55). It is also unlikely that any single medication will fully treat the complex pathophysiology of CF lung disease and highly probable that people with CF will continue to be treated with a combination of drugs in the future. The fact that rhAC is currently being developed as a treatment for patients with Farber disease highlights the potential of re-purposing this drug for CF(56, 57). Towards this end, we have demonstrated that rhAC may be delivered in nebulized form to mice and has important effects *in vivo*.

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Figure Legends

Figure 1 – Ceramide and sphingosine levels in cystic fibrosis and non-cystic fibrosis fully differentiated primary human airway epithelial cell cultures at baseline and in response to *Pseudomonas aeruginosa*. Whole cell lysates of cystic fibrosis (CF) and non-CF cultures at baseline and after co-culture with *Pseudomonas aeruginosa* (PA), levels of total ceramide (a) and sphingosine (b). Radar charts (c) of individual ceramide species (fmole/mg protein). Plasma membrane (PM) fractions of cultures were isolated at baseline and after co-culture with *P. aeruginosa*, allowing determination of total ceramide (d) and sphingosine (e) levels. Individual ceramide species (f), displayed as radar charts (fmole/mg protein). The proportion of ceramide in plasma membranes (as a fraction of total cellular ceramide) is shown in panel (g) and equivalent for sphingosine in (h). Throughout, n=6 separate experiments from individual donors. Cultures were lysed and fractionated into whole cell and plasma membrane fractions after 28 days at air liquid interface and full differentiation. Individual data points are presented along with the mean (horizontal line) and ± standard deviation (error bars). For statistical tests used see the online data supplement *$P<0.05$, **$P<0.01$, ***$P<0.001$.

Figure 2 - Ceramide levels in BAL fluid from children and young people. Levels of ceramide in BAL fluid collected during clinically indicated bronchoscopies from children and young people with cystic fibrosis (CF) and children who do not have CF but underwent a bronchoscopy for investigation of respiratory problems. Groups were matched for age (see Table E1 in the online data supplement). Individual data points are presented along with the mean (horizontal line) and ± standard deviation (error bars). An unpaired t-test was used to determine significance *$P<0.05$.

Figure 3 – Acid ceramidase and acid sphingomyelinase expression and function in cystic fibrosis and non-cystic fibrosis airway epithelial cell cultures. (a) Apical surface activity of ceramidase (Cer), as determined by percentage of fluorescently labeled ceramide processed into sphingosine. (b) Levels of acid ceramidase (AC) protein in cystic fibrosis (CF) and non-CF cultures at baseline and after co-culture with

Pseudomonas. aeruginosa (PA), displayed as change relative to untreated non-CF cultures. Representative blots are shown for AC, (methods for full-length blots are in the online data supplement, with details of loading controls shown in Figure E1). (c) Gene expression of *ASAH1* (coding for acid ceramidase) at baseline in CF and non-CF cultures and after co-culture with *P. aeruginosa*, displayed as fold change relative to untreated non-CF cultures. (d) Apical surface activity of sphingomyelinase (Sph), as determined by percentage of fluorescently labeled sphingomyelin processed into ceramide. (e) Levels of acid sphingomyelinase (ASM) protein, displayed as change relative to untreated non-CF cultures. Representative blots are shown for ASM (methods for full-length blots are in the online data supplement, with details of loading controls shown in Figure E1). (f) Gene expression of *SMPD1* (coding for acid sphingomyelinase), displayed as fold change relative to untreated non-CF cultures. For loading controls, antibody, and primer and reaction details see Fig E1 and Tables E2, E3 and E4 in the online data supplement. Throughout, n=6 separate experiments from individual donors. Individual data points are presented along with the mean (horizontal line) and ± standard deviation (error bars). For statistical tests used see the online data supplement *$P<0.05$, **$P\leq0.01$.

Figure 4 – Effect of recombinant human acid ceramidase treatment on ceramide and sphingosine profile of cystic fibrosis and non-cystic fibrosis airway epithelial cell cultures. Whole cell lysates of cystic fibrosis (CF) and non-CF cultures at baseline and after treatment with recombinant human acid ceramidase (rhAC); levels of total ceramide (a) and sphingosine (b). Individual ceramide species (c), displayed as radar charts (fmol/mg protein). Plasma membrane fractions of CF and non-CF cultures at baseline and after treatment with rhAC - levels of total ceramide (d) and sphingosine (e). Individual ceramide species (f), displayed as radar charts (fmole/mg protein). Proportion of ceramide in plasma membranes (PM) (as a fraction of total cellular ceramide) is shown in panel (g) and the equivalent for sphingosine in (h). Throughout, n=6 separate experiments from individual donors. Cultures were lysed and fractionated into whole cell and plasma membrane fractions after 28 days at air liquid interface and full differentiation. Individual data points are presented along with the mean (horizontal line) and ± standard deviation (error bars). For statistical tests used see the online data supplement *$P<0.05$, **$P\leq0.01$.

Figure 5 – Effect of recombinant human acid ceramidase treatment on inflammatory mediator production by cystic fibrosis and non-cystic fibrosis airway epithelial cell cultures. Time course of apical secretion of (a) IL-8, (b) IL-1β, and (c) TNFα from cystic fibrosis (CF) and non-CF cultures at baseline and after a single treatment with recombinant human acid ceramidase (rhAC). Apical secretion of IL-8 (d) from cultures following pre-treatment with combinations of ivacaftor, tezacaftor-ivacaftor and rhAC. For (a) through (c) n=6 separate experiments, n=4 for (d), CF group F508del/F508del genotype, from individual donors. Data are presented as mean with standard deviation for (a) through (c); for (d) individual data points are presented along with the mean (horizontal line) and ± standard deviation (error bars). For statistical tests used see the online data supplement *$P<0.05$, **$P\leq0.01$, ***$P\leq0.001$ and ns non-significant $P\geq0.05$.

Figure 6 – Effect of recombinant human acid ceramidase treatment on lung inflammation in murine models. Number of (a) neutrophils and (b) macrophages in the submucosa of distal large bronchi in lung sections from wild type (WT), *Cftr*^{ko} and *Cftr*^{MHH} mice at baseline and following nebulization daily for 3 days with recombinant human acid ceramidase (rhAC). Throughout, n=6 mice in each group. Individual data points are presented along with the mean (horizontal line) and ± standard deviation (error bars). For statistical tests used see the online data supplement ***$P\leq0.001$.

Figure 7 – Tumor Necrosis Factor Receptor 1 expression and cRel localization in cystic fibrosis airway epithelial cell cultures and lung tissue sections. Expression of Tumor Necrosis Factor Receptor 1 (TNFR1) in cystic fibrosis (CF) and non-CF cultures with and without recombinant human acid ceramidase (rhAC) treatment as assessed by (a) immunohistochemistry (with quantification of apical mean pixel intensity (MPI) in (b)). cRel expression in CF and non-CF cultures in response to *Pseudomonas aeruginosa* (PA) co-culture in the presence or absence of rhAC in (c) cytoplasmic and (d) nuclear fractions. Representative blots are shown for both (methods are in the online data supplement, with details of loading controls

shown in Figure E1). (e) Apical IL-8 secretion in the presence or absence of a specific cRel inhibitor (Inh). (f) Expression and localization of cRel in airway epithelium in airway tissue sections from people with advanced CF lung disease and unused donor lungs (non-CF) with quantification of (g) whole cell and (h) nuclear localization. For (a) through (d) n=6 separate experiments, n=5 for (e) and n=4 for (f) through (h), all from individual donors, see Table S1 in the online data supplement for clinical details. Data are presented as mean with standard deviation for (b), (g) and (h); for (c) through (e) individual data points are presented along with the mean (horizontal line) and ± standard deviation (error bars). For statistical tests used see the online data supplement *$P<0.05$, **$P\leq0.01$.

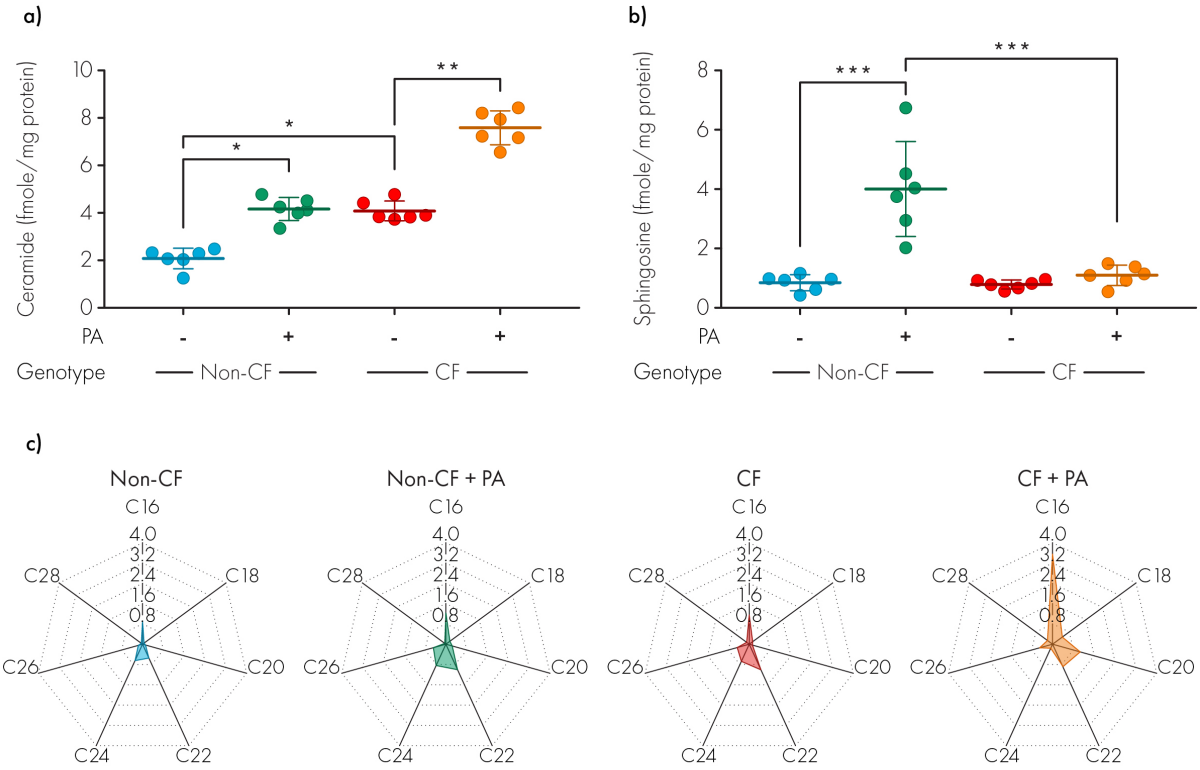
Figure 8 – Effect of recombinant human acid ceramidase treatment on infection in cystic fibrosis airway epithelial cell cultures. Number of fluorescently labeled heat-killed *Staphylococcus aureus* retrieved from apical surface washes (a), and adherent to apical surface (b) in cystic fibrosis (CF) and non-CF fully differentiated cultures with and without prior recombinant human acid ceramidase (rhAC) treatment. For representative images see Fig E6 in the online data supplement. Colony forming unit (CFU) counts of *Pseudomonas aeruginosa* isolated from (c) apical surface washes and (d) whole cell lysates (following washing, suggesting internalization) from CF and non-CF cultures with and without prior rhAC treatment. Live *P. aeruginosa* were added to the apical surface of cultures and allowed to proliferate for 24 hours. Throughout n=6 separate experiments. Individual data points are presented along with the mean (horizontal line) and ± standard deviation (error bars). For statistical tests used see the online data supplement *$P<0.05$, **$P\leq0.01$.

Figure 9 – Proposed model of how altered sphingolipid metabolism in cystic fibrosis airway epithelia may result in increased inflammation and susceptibility to infection. (a) In non-cystic fibrosis epithelia acid ceramidase (AC) maintains the balance of ceramide (Cer.) and sphingosine (Sph.). Normal levels of ceramide do not promote a pro-inflammatory environment and in response to *P. aeruginosa* levels of sphingosine are up-regulated. (b) In cystic fibrosis (CF) epithelia AC activity is deficient

in both expression and activity which, in combination with alterations in acid sphingomyelinase activity, leads to the accumulation of ceramide. Raised ceramide is associated with increased tumor necrosis factor receptor 1 (TNFR) expression, enhanced NF-κB activation and nuclear localization of cRel. This promotes the secretion of pro-inflammatory cytokines such as IL-8, IL-1β and TNFα. In conjunction with excessive recruitment of immune cells, which also produce pro-inflammatory mediators, a positive feedback loop emerges in the cystic fibrosis airway. Cystic fibrosis epithelia do not up-regulate sphingosine in response to *P. aeruginosa,* increasing susceptibility to infection, which further contributes to the pro-inflammatory environment. Treatment with recombinant human acid ceramidase (rhAC) reduces ceramide and increases sphingosine, ameliorating these effects.

Figures

Figure 1 – Ceramide and sphingosine levels in cystic fibrosis and non-cystic fibrosis fully differentiated primary human airway epithelial cell cultures at baseline and in response to *Pseudomonas aeruginosa*.



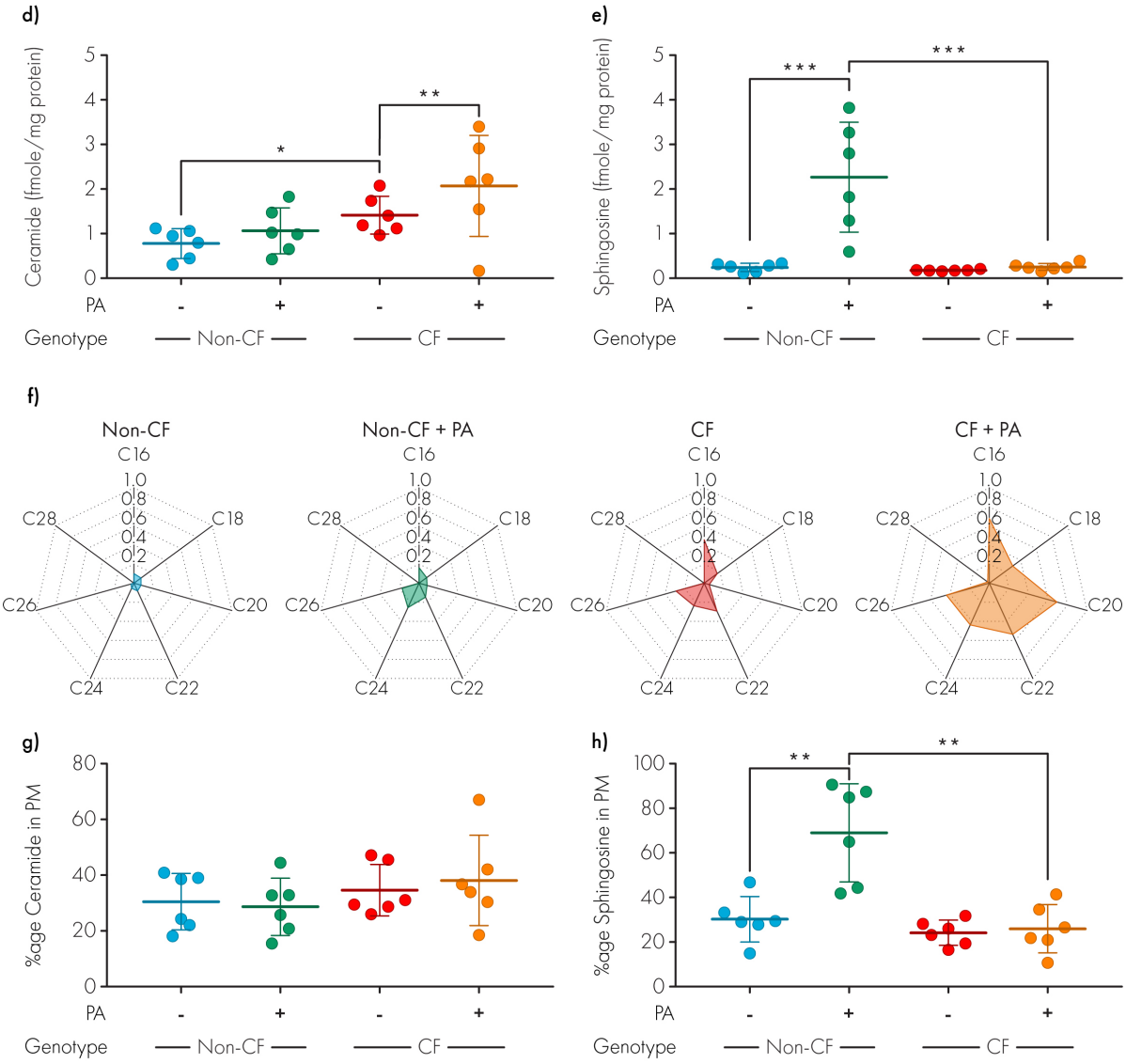

d)
Ceramide (fmole/mg protein)
PA
Genotype
Non-CF
CF
e)
Sphingosine (fmole/mg protein)
f)
Non-CF
Non-CF + PA
CF
CF + PA
C16
C18
C20
C22
C24
C26
C28
g)
%age Ceramide in PM
h)
%age Sphingosine in PM

Figure 2 - Ceramide levels in BAL fluid from children and young people.

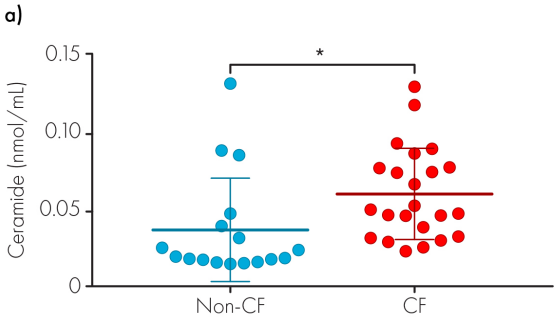


Figure 3 – Acid ceramidase and acid sphingomyelinase expression and function in cystic fibrosis and non-cystic fibrosis airway epithelial cell cultures.

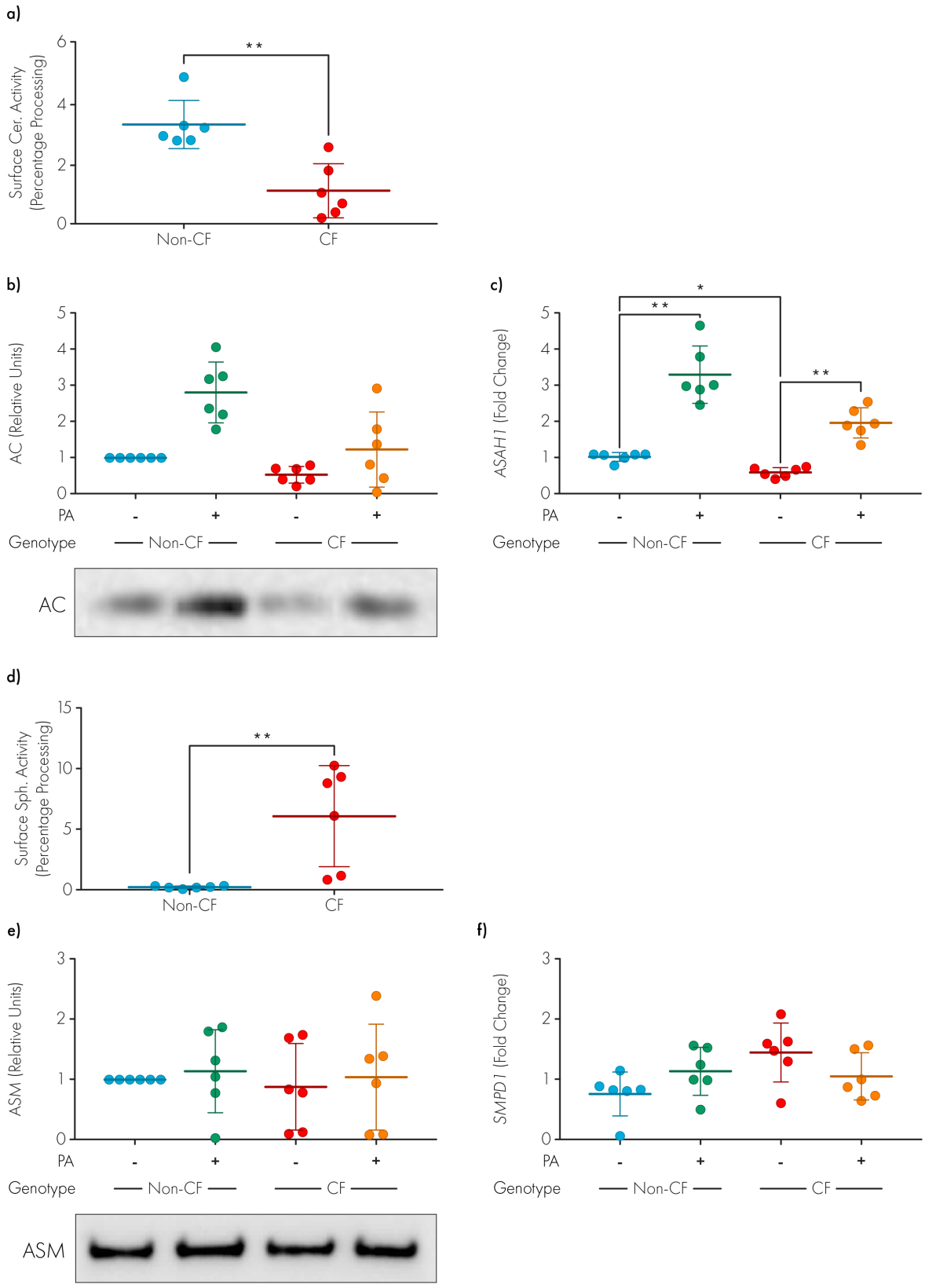
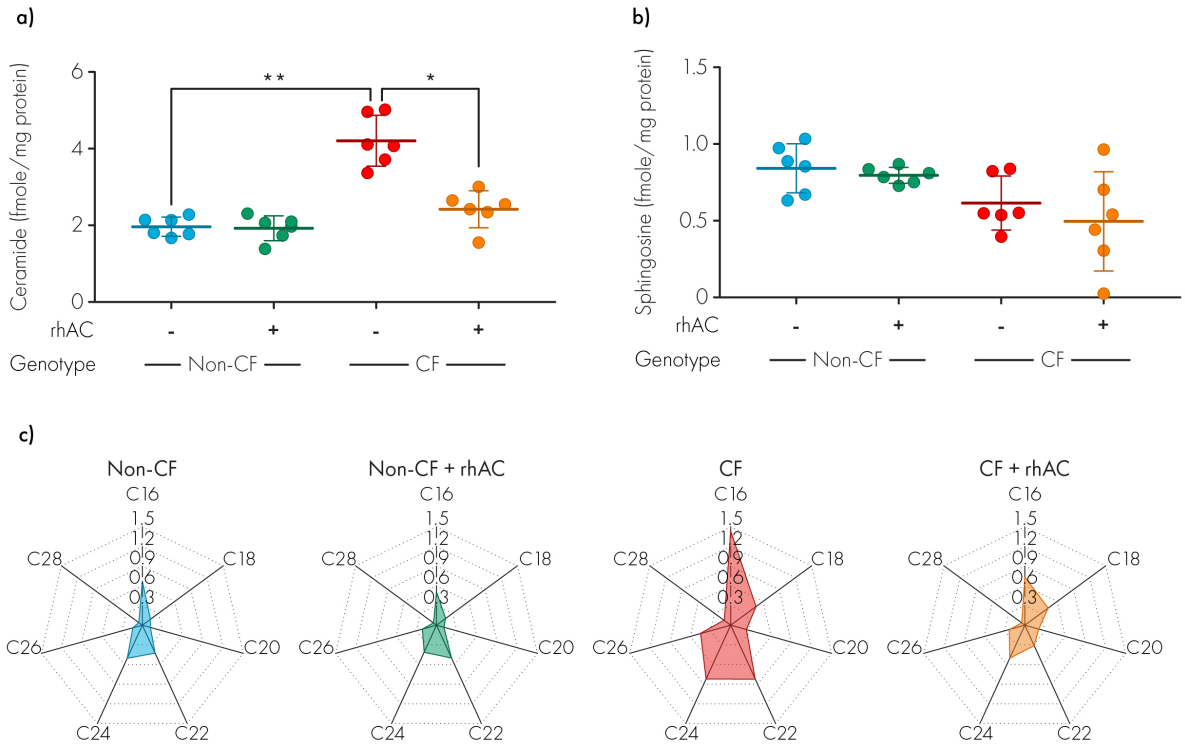
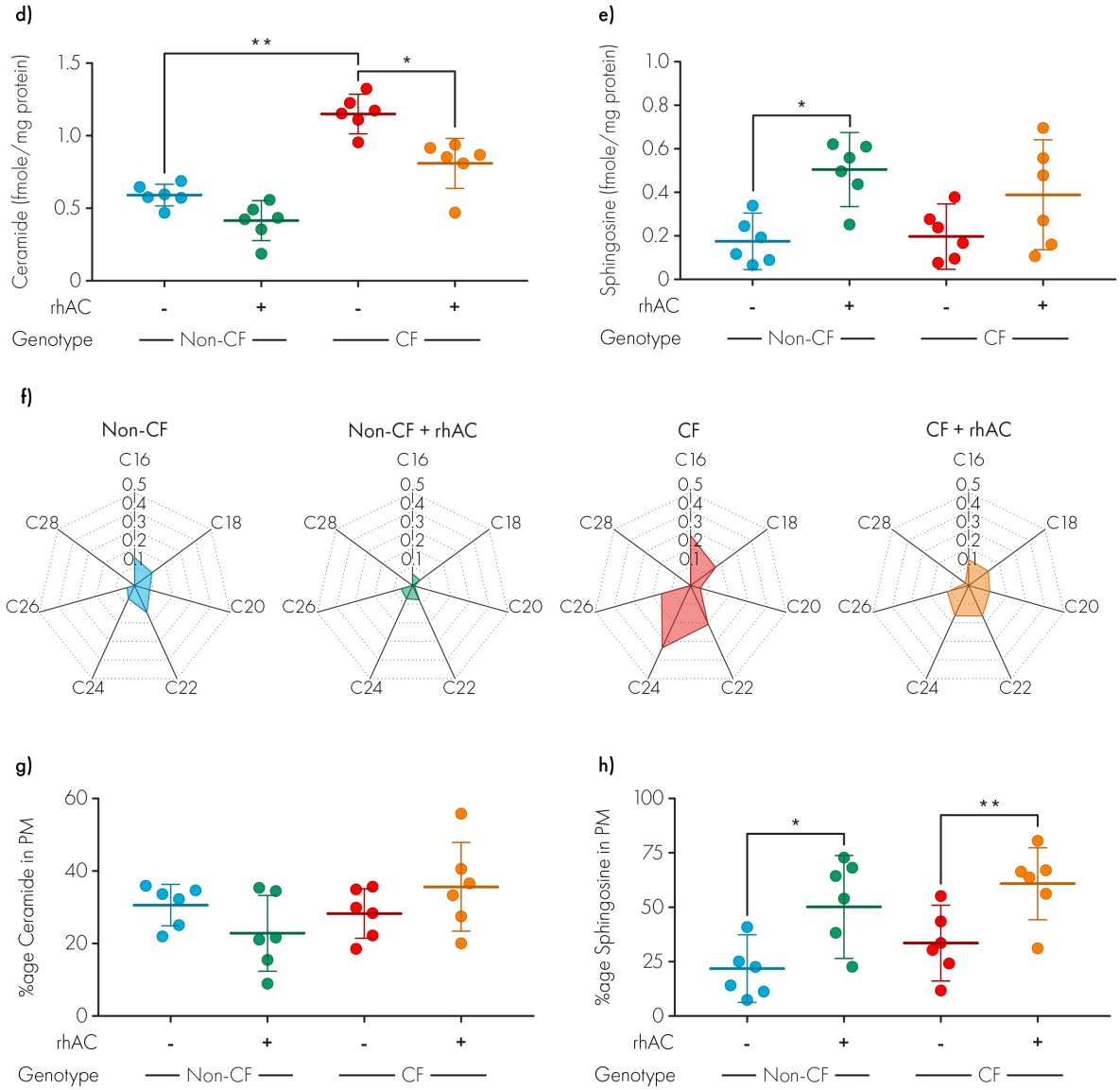


Figure 4 – Effect of recombinant human acid ceramidase treatment on ceramide and sphingosine profile of cystic fibrosis and non-cystic fibrosis airway epithelial cell cultures.




d)
Ceramide (fmole/mg protein)
1.5
1.0
0.5
0
**
*
rhAC
-
+
-
+
Genotype
Non-CF
CF
e)
Sphingosine (fmole/mg protein)
1.0
0.8
0.6
0.4
0.2
0
*
rhAC
-
+
-
+
Genotype
Non-CF
CF
f)
Non-CF
Non-CF + rhAC
CF
CF + rhAC
C16
C18
C20
C22
C24
C26
C28
0.5
0.4
0.3
0.2
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g)
%age Ceramide in PM
60
40
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Non-CF
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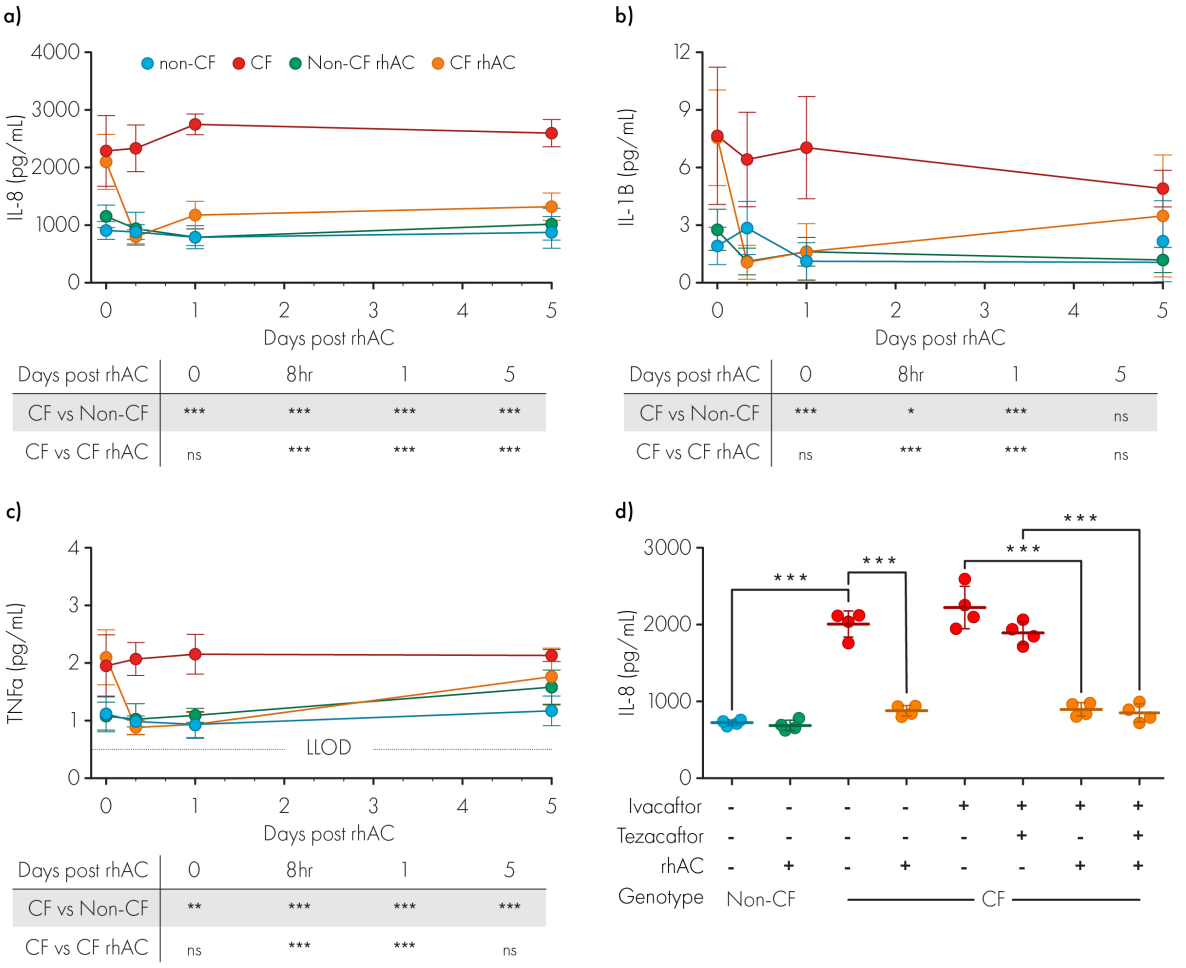
Figure 5 – Effect of recombinant human acid ceramidase treatment on inflammatory mediator production by cystic fibrosis and non-cystic fibrosis airway epithelial cell cultures.



Days post rhAC	0	8hr	1	5
CF vs Non-CF	***	***	***	***
CF vs CF rhAC	ns	***	***	***

Days post rhAC	0	8hr	1	5
CF vs Non-CF	***	*	***	ns
CF vs CF rhAC	ns	***	***	ns

Days post rhAC	0	8hr	1	5
CF vs Non-CF	**	***	***	***
CF vs CF rhAC	ns	***	***	ns

Figure 6 – Effect of recombinant human acid ceramidase treatment on lung inflammation in murine models.

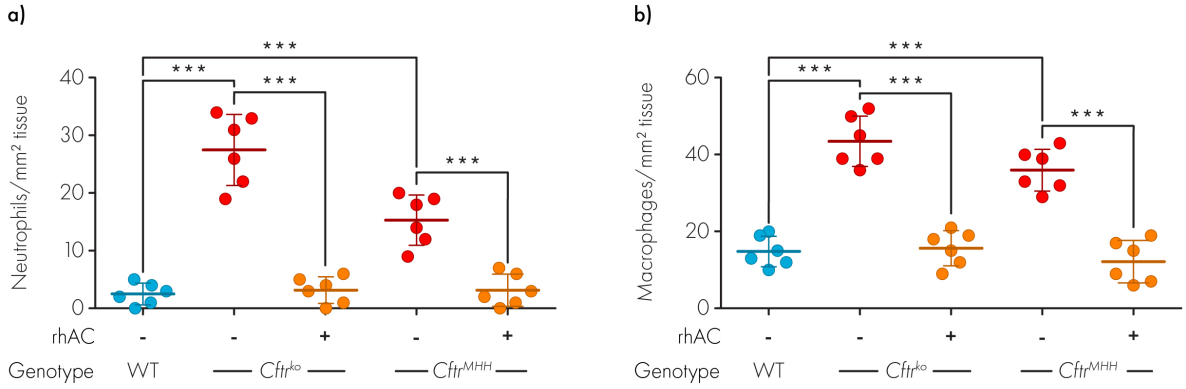
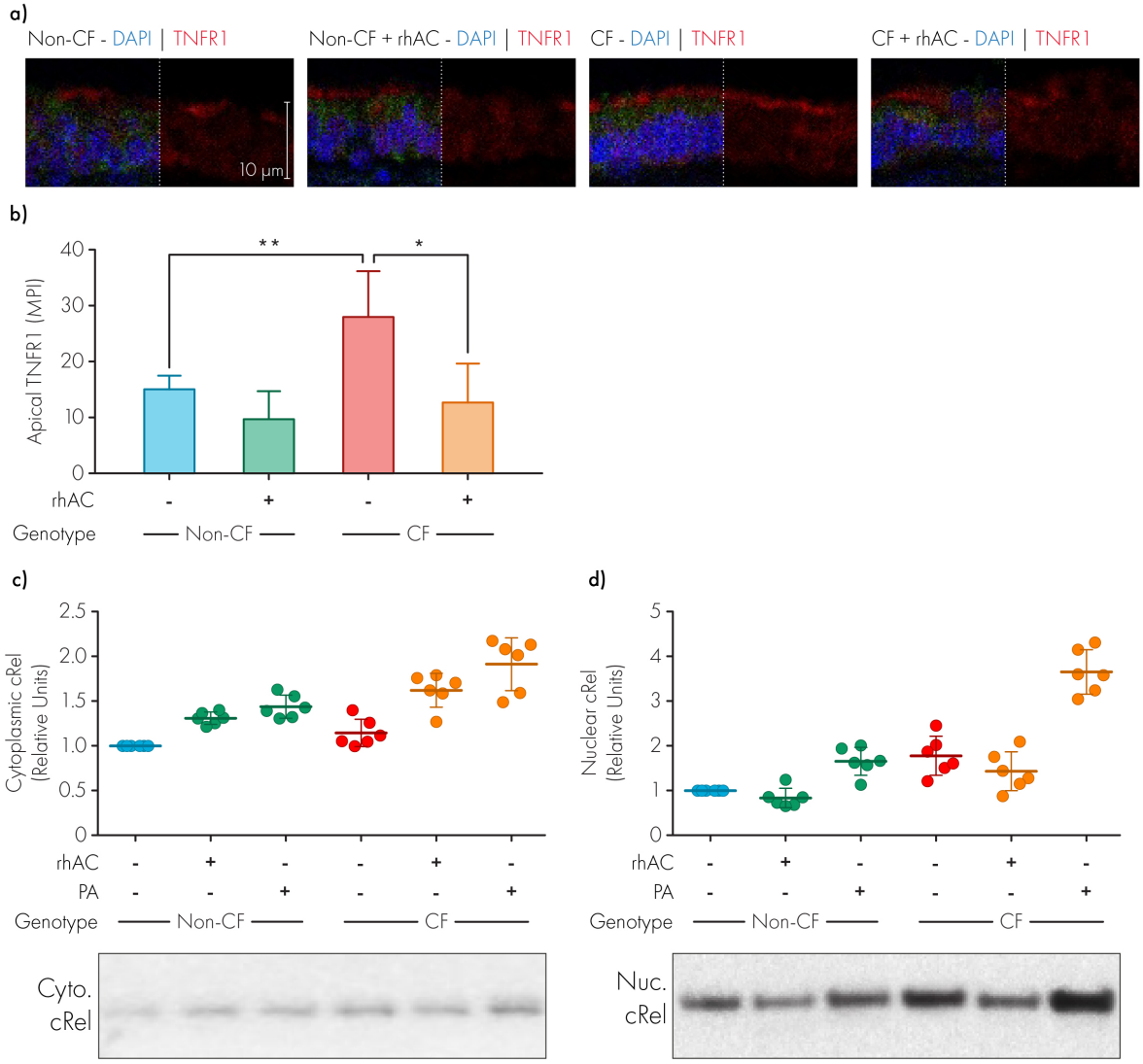
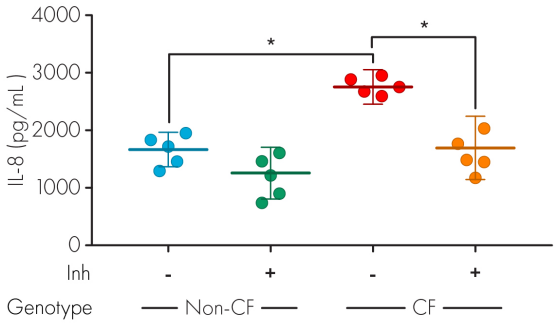


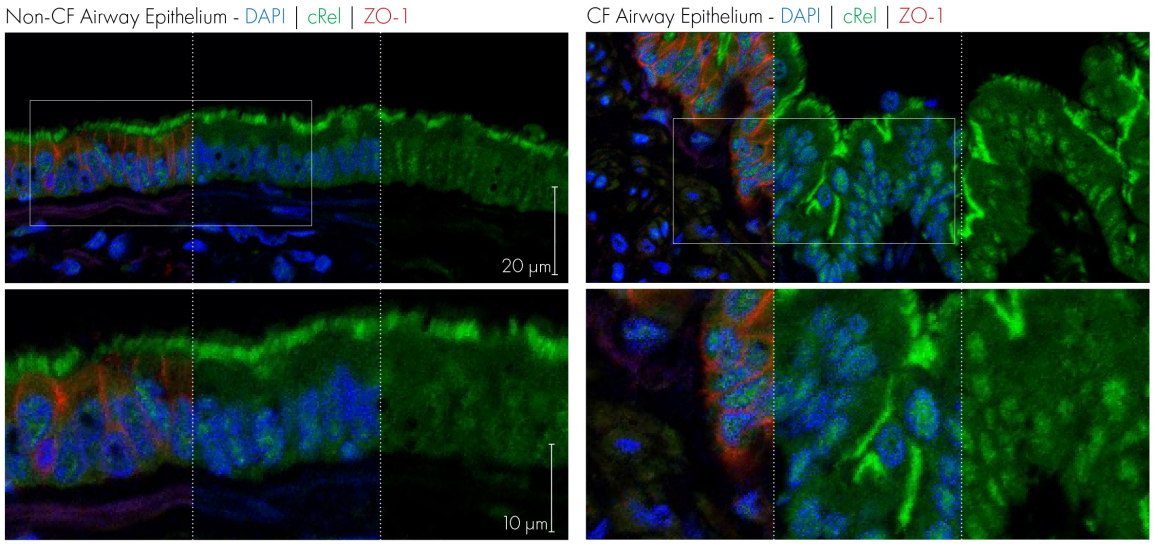
Figure 7 – Tumor Necrosis Factor Receptor 1 expression and cRel localization in cystic fibrosis airway epithelial cell cultures and tissue sections



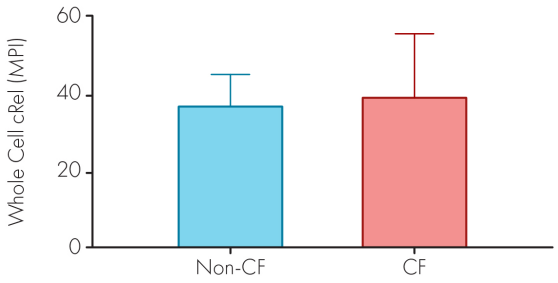
e)


4000
3000
2000
1000
0
IL-8 (pg/mL)
*
*
Inh
-
+
-
+
Genotype
Non-CF
CF

f)


Non-CF Airway Epithelium - DAPI | cRel | ZO-1
CF Airway Epithelium - DAPI | cRel | ZO-1
20 µm
10 µm

g)


60
40
20
0
Whole Cell cRel (MPI)
Non-CF
CF

h)

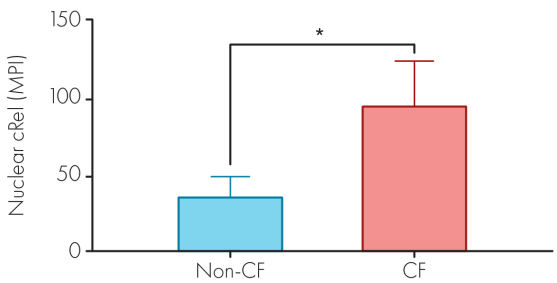

150
100
50
0
Nuclear cRel (MPI)
*
Non-CF
CF

Figure 8 – Effect of recombinant human acid ceramidase treatment on infection in cystic fibrosis airway epithelial cell cultures.

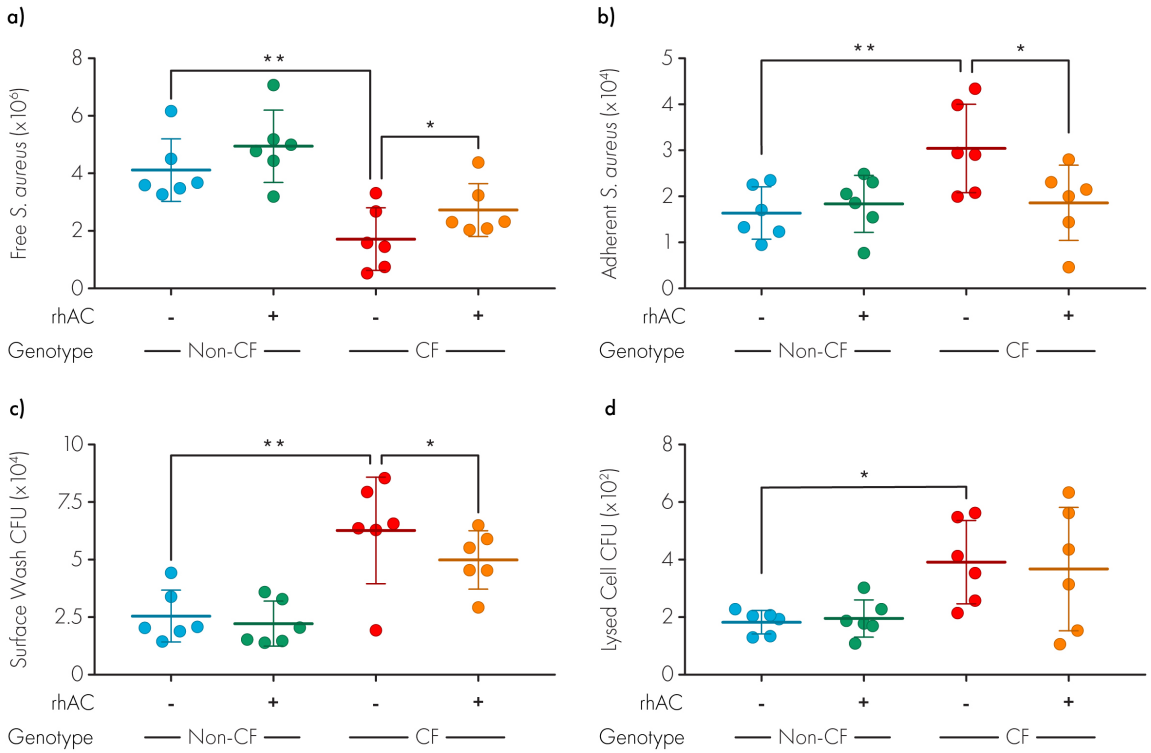


Figure 9 – Proposed model of how altered sphingolipid metabolism in cystic fibrosis airway epithelia may result in increased inflammation and susceptibility to infection.